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Influence of zinc on CTX-M-1 β-lactamase expression in *Escherichia coli*

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ABSTRACT

Objective: Zinc oxide is used to prevent post-weaning diarrhoea in pigs as an alternative to antimicrobial growth promoters. This study aims to determine if the use of zinc oxide selects for extended-spectrum β-lactamase (ESBL)-producing *Escherichia coli* and affects the expression of $bla_{CTX-M-1}$ in *E. coli*.
Methods: Using an in vitro faecal micro-cosmos model, the selective properties of zinc were investigated using an *E. coli* strain with $bla_{CTX-M-1}$ encoded by a natural IncI1 resistance plasmid (MG1655/pTF2) and another strain where the same gene was located on the chromosome (MG1655::$bla_{CTX-M-1}$). The micro-cosmos was seeded with faecal material containing an increasing concentration of zinc (0–8 mM). Outcome measurements consisted of colony-forming units (CFU) of the inoculated ESBL *E. coli* and naturally occurring coliforms as determined by plate counting on MacConkey with and without 5 mg/L cefotaxime as well as total viable bacteria determined on Luria agar without cefotaxime. Expression of $bla_{CTX-M-1}$ under the experimental zinc concentrations was determined by quantitative polymerase chain reaction.
Results: The proportion of MG1655/pTF2 of the total viable bacteria was significantly higher at high zinc concentrations (6 and 8 mM) compared with low concentrations (0–4 mM). The messenger RNA (mRNA) levels of $bla_{CTX-M-1}$ in the two ESBL strains increased at increasing zinc concentrations and varied with the growth phase.
Conclusion: The growth of the inoculated CTX-M-1-encoding *E. coli* MG1655 strains and naturally occurring coliforms was impacted differently when exposed to zinc oxide. The $bla_{CTX-M-1}$ mRNA expression levels seemed to increase with increasing zinc concentrations, but varied with growth phase, but not gene location.

1. Introduction

Antimicrobial resistance is a worldwide health problem with serious consequences on mortality and morbidity of infectious diseases as well as a financial burden on healthcare systems [1]. If proper actions are not taken, the prediction is that we will soon face untreatable infections [2]. Owing to the emergence of multi-resistant bacteria in animal production and the risk of transfer of resistant bacteria to humans [3,4], the use of antimicrobial growth promoters has been banned in the European Union since 2006 [5]. Several non-antimicrobial substances have been considered as alternative feed additives to promote growth and decrease the pathogen load in animals, including prebiotics, probiotics, enzymes and cationic trace elements such as zinc oxide [6,7].

Zinc is an essential element, naturally present in food and feed. It is involved in various physiological functions, and it is a cofactor for more than 300 enzymes covering all six classes of enzymes [8,9]. As such, zinc is necessary for cell division and DNA synthesis in bacteria [10]. Dietary zinc is used to increase growth in weanling pigs, but in some countries, zinc oxide is also incorporated into the post-weaning diet at therapeutic levels (between 2.5 and 3.1 g/kg) to prevent or reduce the severity of post-weaning diarrhoea (PWD), and thus improve growth performance [11–13]. Owing to adverse environmental effects, however, the use of zinc for therapeutic purposes will be phased out in Europe from 2022 [14].

High levels of zinc oxide supplementation may increase the occurrence of antimicrobial resistance and promote multi-resistance development in bacteria from animals and in animal excretions via co-selection and/or other mechanisms [10,15–17]. Thus, the use of zinc oxide as a feed supplement has been associated with increased resistance against antimicrobials such as

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penicillin, ampicillin, tetracycline and sulfonamides in pigs [18]. Zinc may also promote the occurrence of extended-spectrum β-lactamase (ESBL)-producing *Escherichia coli*, causing hard-to-treat infections in humans and animals [4,16]. There are diverse types of ESBLs, and the most frequently detected in *E. coli* are of the classes CTX-M encoded by $bla_{\text{CTX-M}}$ (hydrolyses cefotaxime [CTX]), TEM encoded by bla_{TEM} (named after the patient Temoniera) and SHV encoded by bla_{SHV} (sulfhydryl reagent variable) [19]. In Europe, CTX-M-1 is the most common type found in livestock [20–22] and the second most reported from human isolates in countries such as Italy and France [23].

Studies have investigated the effect of dietary zinc oxide on *E. coli* diversity and antimicrobial resistance in the animal gut [4,17,24,25], pig manure [10] and pig production [26]. These studies used animal experiments and excreta analysis. However, little is known about how zinc oxide selects for ESBL-producing *E. coli* and expression of the CTX-M-encoding genes. In this study, we investigated (i) the growth response of CTX-M-1-encoding *E. coli* exposed to different concentrations of zinc oxide and (ii) how zinc oxide influences messenger RNA (mRNA) expression profiles of $bla_{\text{CTX-M-1}}$.

2. Methods

2.1. Growth of ESBL-producing E. coli in micro-cosmos at different zinc concentrations

An in vitro faecal micro-cosmos (suspension of 10% g/mL pig faeces in buffered peptone water and homogenized in a stomacher) was used. Pig faeces were obtained from a company raising pigs for animal experiments in which pigs had not been treated with antimicrobials and/or zinc oxide. The selective properties of zinc for ESBL-producing bacteria were investigated using two *E. coli* strains, MG1655 carrying $bla_{\text{CTX-M-1}}$ on a naturally occurring IncI1 resistance plasmid (MG1655/pTF2) and MG1655 carrying the same gene on the chromosome (MG1655::$bla_{\text{CTX-M-1}}$). The origin of the strains has previously been described [27].

A concentration of 10^6 colony-forming units (CFU)/mL of the two test strains was seeded in each experiment to the micro-cosmos at increasing zinc chloride ($ZnCl_2$) concentrations of 0, 1, 2, 4, 6 and 8 mM (equals ca. 3 g/kg feeding dose as a result of 1:5 dilution of feed in small intestinal chyme [28]), and growth was monitored after incubation at 37 °C for 24 h. We used $ZnCl_2$ to replace zinc oxide which is typically used by farmers, because zinc oxide has higher solubility at acidic conditions owing to low stomach pH [28], and its solubility is increased after feed intake and then, soluble Zn^{2+} ions (mainly as $ZnCl_2$ as a result of hydrochloric acid) can be observed [29,30]. The outcome measurements consisted of CFU of ESBL *E. coli*, coliforms and total viable bacterial counts determined by plate counting on MacConkey agar (Oxoid, Copenhagen, Denmark) supplemented with 5 mg/L CTX (Sigma, Copenhagen, Denmark) (when *E. coli* MG1655 was added) or without CTX (control for determination of total coliforms, with no added test strains) and Luria agar (enumeration of total viable bacteria) (Oxoid) without CTX at 0, 6, 12 and 24 h after incubation of the test strains. The experiments were done in triplicate within 1 week using the same faecal material.

2.2. Antimicrobial susceptibility testing

The minimal inhibitory concentration (MIC) of CTX was determined using the broth microdilution method in accordance with the Clinical and Laboratory Standards Institute (CLSI) guidelines [31]. Mueller-Hinton II (MH-2) (Sigma) broth supplemented with CTX was inoculated with each *E. coli* MG1655 bacterial suspension of 10^5 CFU/mL and incubated aerobically without shaking at 37 °C for 24 h. The CTX concentrations tested ranged from 0 to 512 mg/L by two-fold dilution increase [27].

2.3. Zinc susceptibility testing

Zinc susceptibilities of both *E. coli* MG1655 strains were tested by the agar dilution method with 20 μL of suspension of bacterial cultures (1×10^6 CFU/mL) added onto the surface of MH-2 agar plates supplemented with increasing concentrations (0, 0.5, 1.0, 2.0, 4.0 and 6.0 mM) of $ZnCl_2$ (pH = 7.1) and incubated at 37 °C for 24 h. As there is no approved interpretative standard available for the classification of *E. coli* as susceptible or resistant to zinc, we set the threshold to 6 mM $ZnCl_2$ based on recently published values [4,28].

2.4. Growth under $ZnCl_2$ supplemented conditions

Growth experiments were conducted in triplicate on a BioScreen CTM (Oy Growth Curves Ab Ltd., Finland) for 24 h at 37 °C. A volume of 200 μL of MH-2 broth was inoculated with bacteria cultured on Blood Agar Base supplemented with 5% blood from cattle (Hatunalab, Malaren, Sweden) to a final cell density of 10^6 CFU/mL, using a Sensititre™ Nephelometer (Thermo Scientific™, Copenhagen, Denmark) with a 0.5 McFarland standard ($1–2 \times 10^8$ CFU/mL). The culture in MH-2 broth was supplemented with $ZnCl_2$ ranging from 0 to 4 mM by two-fold dilutions based on the results from our pilot experiments with zinc susceptibility testing. The optical density (OD) was measured every 5 min with continuous shaking (recorded with a 600-nm filter). The Hill coefficient of each growth curve was calculated using a non-linear model of the log-transformed OD_{600} values applying GraphPad Prism 7 (GraphPad Software, CA, USA) [27].

2.5. Expression of $bla_{\text{CTX-M-1}}$

For analysis of the expression of $bla_{\text{CTX-M-1}}$, the two test strains were grown in 250 mL flasks containing 100 mL of MH-2 broth at 37 °C and shaking at 225 rpm. The medium was supplemented with increasing concentrations of zinc, i.e. 0, 0.25, 0.5 and 1 mM. The concentrations were defined based on the results from the zinc susceptibility testing and the pilot experiments.

Extraction of RNA was done following a recently published study [27] and the manufacturer's instructions. In brief, samples for RNA extraction were collected at three times during the in vitro growth experiment: the logarithmic phase (OD_{600} = 0.5–0.6), the late logarithmic phase (OD_{600} = 1–1.3) and the stationary phase (OD_{600} = 3.3–4.6) [27]. Quantitative polymerase chain reaction was conducted using a LightCycler 96 (Roche, Hvidovre, Denmark), essentially as described by Pfaffl [32]. We used the same primer sequences as described by Kjeldsen et al. [27]. The genes *gap*A and *nus*G were selected as a reference, and the relative gene expression was calculated compared with the logarithmic phase sample of MG1655/pTF2 cultured without zinc. The $2^{-\Delta\Delta Ct}$ method corrected for different primer efficiencies and multiple reference genes was used [32].

2.6. Statistical analysis

The analyses were performed using SPSS version 25 (IBM Corporation), and differences in least-squares mean estimates were assessed with *t* test. The χ^2 test was used to determine if differences in the ratio existed in the corresponding time point of the two test strains and values of $P < 0.05$ was regarded as statistically significant. The Brown-Forsythe test was used to confirm the homogeneity of variances when conducting multiple comparisons. The differences in normalized quantitative

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polymerase chain reaction measurements between zinc concentrations with each growth were compared by differences in least-squares means applying the analysis of variance stratified by bacterial strain. The *F*-test was also used to test whether differences existed between concentrations and growth phases. The Benjamini-Hochberg 'false discovery rate' approach was used to correct for multiple comparisons of the differences in the least-squares means ($P < 0.05$) [33,34].

3. Results

3.1. ESBL E. coli growth in the faecal micro-cosmos at different zinc concentrations

No colonies were observed on MacConkey agar plates with CTX at 5 mg/L when 100 μL of 1/10 dilution of faecal material was spread. The MICs of CTX were 256 and 128 mg/L for MG1655/pTF2 and MG1655::$bla_{CTX\text{-}M\text{-}1}$, respectively, and the MIC of zinc was 2 mM for both strains. The two selected strains were confirmed to be able to grow on MacConkey agar plates with 5 mg/L CTX in our pilot experiments (data not shown).

The total number of bacteria estimated on Luria agar (B0–B5) was not significantly affected by the zinc concentration and bacteria added (Fig. 1). As shown in Fig. 1A, the growth of MG1655/pTF2 (A0–A5) was different depending on the zinc concentration at 12 h, where a significantly higher number of colonies was observed at the two highest zinc concentrations (A4 and A5) compared to the low concentrations (A0–A3) ($P < 0.05$). However, no statistical significance was observed at 24 h post-inoculation.

For the MG1655 strain with chromosomal encoded $bla_{CTX\text{-}M\text{-}1}$ (MG1655::$bla_{CTX\text{-}M\text{-}1}$; Fig. 1B), the numbers of bacteria (A0–A5) were not significantly different depending on the zinc concentration. However, the overall counts of the naturally occurring coliforms decreased significantly in samples with the highest zinc concentrations (A5) compared with those with the lower zinc concentrations (A1) at 6 h and 12 h in the control group (Fig. 1C). The slope of the growth curves for each strain at each concentration from 0 to 6 h was not significantly different (Supplementary Figs. S1 and S2). The ratio of MG1655/pTF2 counts to total viable bacteria decreased up to time point 6 h with increasing concentrations of zinc, and the ratios were significantly higher for the two highest zinc concentrations (R4 and R5) than seen at the four lower zinc concentrations (R0–R3) at time point 12 h ($P < 0.05$) (Supplementary Fig. S4A). In contrast, the ratio of coliforms to the total flora generally decreased gradually with increasing concentrations of zinc, and ratios of the two lowest zinc concentrations (R0 and R1) differed significantly from the ratio of the two highest zinc concentrations (R4 and R5) at time point 6 h, and from that of the highest zinc concentration (R4 and R5) at point 12 h ($P < 0.05$) (Supplementary Fig. S4C).

3.2. Growth of the E. coli MG1655 under different concentrations of zinc

To determine the suitable concentration of zinc concentrations for expression studies, the growth of the MG1655 strains was determined in MH2 media in the presence of increasing concentrations of zinc (0–4 mM). The two isolates showed similar growth patterns and did not grow in MH2 media at the three highest concentrations of $ZnCl_2$ tested (1.5, 2 and 4 mM) (Fig. 2). Also, a significant delay in growth (defined as the time needed to reach OD_{600} = 0.8) was observed for both strains when the media was supplemented with $ZnCl_2$ at 0.5 and 1 mM compared with the growth in media without zinc ($P < 0.01$).

3.3. $bla_{CTX\text{-}M\text{-}1}$ mRNA levels depend on zinc concentration, growth phase and gene location

Based on the results above, expression of $bla_{CTX\text{-}M\text{-}1}$ in the two strains was measured from 0 to 1 mM $ZnCl_2$ (Fig. 3). The increase in

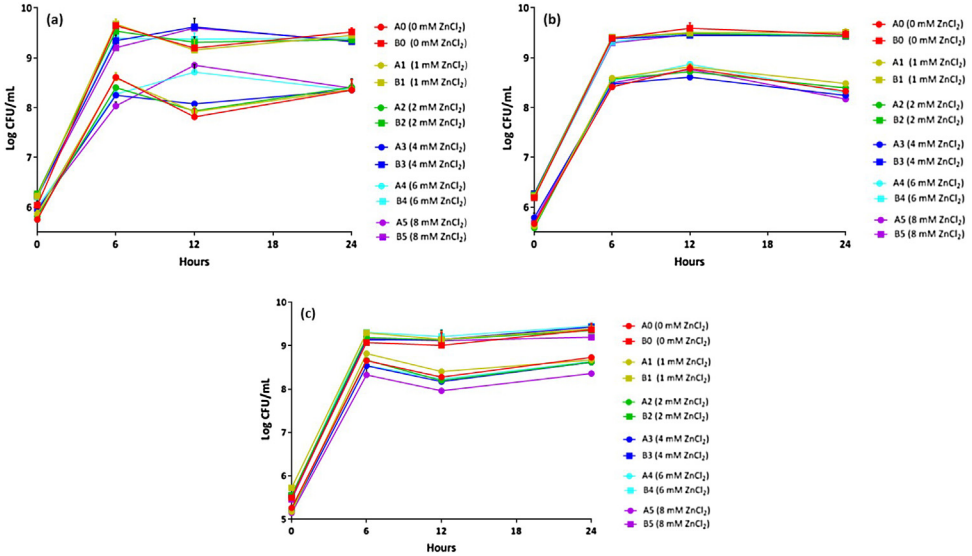

Fig. 1. Bacterial counts in the faeces micro-cosmos. (A) Faeces suspension with added strain MG1655/pTF2 counted on MacConkey agar plates with cefotaxime (CTX) and Luria agar (LA) plates without CTX at different time points. A0–A5 show the number of colonies counted on MacConkey agar plates with CTX corresponding to the numbers obtained for the specific *Escherichia coli* MG1655 strain, and B0–B5 are the number of colonies on LA plates without CTX (total number of viable bacteria). (B) Faeces suspension with added strain MG1655::$bla_{CTX\text{-}M\text{-}1}$ counted on MacConkey agar plates with CTX and LA plates without CTX at different time points. A0–A5 represent the number of colonies counted on MacConkey agar plates with CTX corresponding to the numbers obtained for the specific *E. coli* MG1655 strain, and B0–B5 were the numbers on LA plates without CTX (total number of viable bacteria). (C) The results from the control experiment are shown; the original faeces micro-cosmos without added strains and A0–A5 represent the number of bacteria counted on MacConkey agar (total number of coliforms), and B0–B5 show the number of colonies on LA plates (total number of bacteria). The faeces were diluted 10% in buffered peptone water and then homogenized in a stomacher. Faecal suspensions were supplemented with $ZnCl_2$ at different concentrations (A0/B0, A1/B1, A2/B2, A3/B3, A4/B4 and A5/B5 at zinc concentrations of 0, 1, 2, 4, 6 and 8 mM, respectively). Assays were performed in triplicate. The data shown represent the mean, and error bars represent standard deviations.

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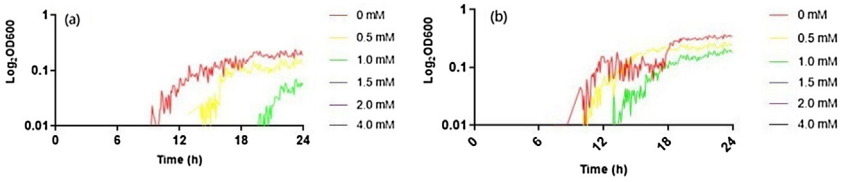

Fig. 2. Growth curves obtained for *Escherichia coli* MG1655 CTX-M-1 in the presence of different concentrations of zinc. Each strain was grown in Mueller-Hinton II (MH-2) broth on a BioScreen CTM. (A) Growth observed for *E. coli* MG1655 containing $bla_{CTX\text{-}M\text{-}1}$ on an IncI1 plasmid (MG1655/IncI1/CTX-M-1) and (B) growth observed for *E. coli* MG1655 containing $bla_{CTX\text{-}M\text{-}1}$ on the chromosome (MG1655/CTX-M-1). All experiments were performed in triplicate. The data shown represent mean values. It should be noted that no growth was observed for both strains at zinc concentrations over 1.5 mM.

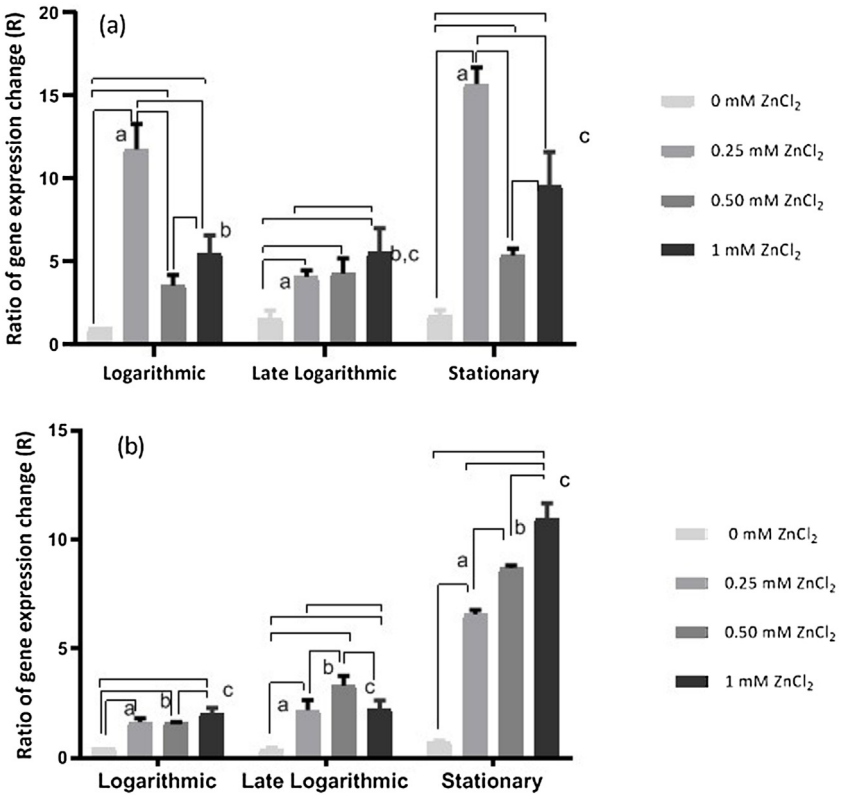

Fig. 3. Relative changes in gene expression of $bla_{CTX\text{-}M\text{-}1}$ messenger RNA (mRNA) in (A) MG1655/pTF2 containing $bla_{CTX\text{-}M\text{-}1}$ on a plasmid and (B) MG1655::$bla_{CTX\text{-}M\text{-}1}$. Strains were grown in Mueller-Hinton II (MH-2) broth media without and with zinc at different concentrations. Assays were performed in triplicate; the data shown represent the mean with standard deviations. The data are relative to the $bla_{CTX\text{-}M\text{-}1}$ mRNA in the logarithmic phase with no zinc for MG1655/IncI1/CTX-M-1. Identical letters in each graph indicate significant growth-phase differences between the two samples ($P < 0.05$), and lines between bars indicate significant differences between the two samples ($P < 0.05$).

mRNA correlated with increasing zinc concentrations, but in a growth phase and strain-dependent manner. Significantly higher levels of $bla_{CTX\text{-}M\text{-}1}$ mRNA were observed for both strains when grown with zinc in all three growth phases compared to growth in media without zinc, and mRNA levels increased with increasing zinc concentrations, except for the $bla_{CTX\text{-}M\text{-}1}$ mRNA levels at 0.25 mM zinc in the logarithmic and stationary phases for MG1655/pTF2, and 0.25 mM in the logarithmic phase and 1 mM in the late logarithmic phase for MG1655::$bla_{CTX\text{-}M\text{-}1}$.

Significant differences in mRNA levels were observed between the three growth phases within all zinc concentration series for MG1656::$bla_{CTX\text{-}M\text{-}1}$; however, for MG1655/pTF2, this was not the case at 0.5 mM zinc. The total mRNA level did not differ significantly between the two strains ($P = 0.66$).

4. Discussion

In this study, we investigated the growth response of two CTX-M-1-producing variants of *E. coli* MG1655 to zinc in an in vitro faecal micro-cosmos over 24 h, as well as the effect of different zinc concentrations on $bla_{CTX\text{-}M\text{-}1}$ expression. We demonstrated that the proportion of a CTX-M-1-resistant *E. coli* increased compared with the total flora when high concentrations of zinc were added to the faecal suspensions. However, this was only observed for the MG1655 strain where $bla_{CTX\text{-}M\text{-}1}$ was located on a plasmid. Thus, the growth advantage cannot be concluded to be related to the $bla_{CTX\text{-}M\text{-}1}$ gene per se, but to the presence of the IncI1 plasmid. The majority of CTX-M encoding Enterobacteriaceae, however, has the $bla_{CTX\text{-}M\text{-}1}$ gene located on plasmids [35,36], with IncI1 plasmids as the most common [35,37], and as such one may fear that they often have a growth advantage in the presence of zinc. The naturally occurring coliform bacteria, however, was reduced relatively to the total viable bacteria at these high zinc concentrations. We also showed that $bla_{CTX\text{-}M\text{-}1}$ mRNA expression increased with increasing zinc concentrations in a growth phase-dependent manner for both strains. These results suggested that high zinc oxide concentrations, as used therapeutically in weaning pigs, might not only give a selective growth advantage to plasmid-encoded ESBL *E. coli*, but also may induce expression of resistance genes in these strains. The mechanism behind this is currently unknown, but it would be

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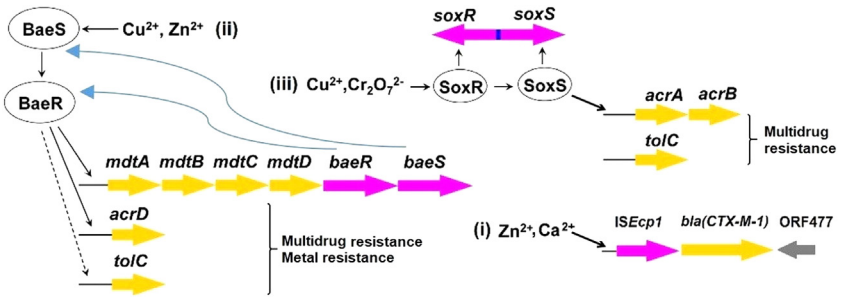

Fig. 4. Possible mechanisms for how heavy metals (e.g. zinc and copper) can induce the expression of resistance genes. (i) The transposase expression of insertion sequence (IS) element (IS*Ecp1*) is zinc-responsive. (ii) The two-component system (BaeS/BaeR) activates expression of the *mdt* operon, *acrD* and *tolC* and confers multidrug and metal resistance. (iii) The global regulator SoxS activates *acrAB* expression in response to copper ions.

interesting to determine whether this is specific to the $bla_{\text{CTX-M-1}}$ gene investigated here or a general phenomenon for several β-lactamases.

In our study, the results of bacterial growth at different zinc concentrations revealed that zinc could inhibit the growth of natural coliforms from the faecal micro-cosmos. This might be because zinc is toxic at high concentrations, as it can interact with thiols and block essential reactions in the cell [38–40], resulting in the growth inhibition of natural coliforms in faecal materials. Interestingly, zinc did not inhibit the growth of MG1655::$bla_{\text{CTX-M-1}}$ and even promoted the growth of MG1655/pTF2 at high concentrations. Our results were based on CFU counts on MacConkey agar, and we have not confirmed that colonies were indeed the original MG1655 strains; however, the faecal microflora used was the same as in the control batches, where no increase in growth was observed. It has been reported not only that the growth of strain LSJC7 (a Gram-negative member of the family Enterobacteriaceae with resistance to arsenic and tetracycline) was significantly promoted in the presence of zinc or copper [41], which corresponds to our observations, but also that the presence of metal stress (like from Zn and Cu) was shown to increase plasmid mobilization capacity [42,43]; thus, metal stress may increase the permissiveness of different members of a bacterial community towards a plasmid [44], and we cannot rule out that part of the increasing CFU of the CTX-M-encoding strain with pTF2 is because of plasmid transfer promoted by high zinc concentrations.

The results of growth advantage may be explained by co-regulation as well, i.e. the transcriptional and translational responses to antimicrobial or zinc exposure, and they can be linked to a form of a coordinated response to either stress. The efflux system regulated by *mdt*ABC operon has been involved in conferring resistance to some certain antimicrobials [45,46] and it is widely present in ESBL *E. coli*. Lee et al. [45] demonstrated that the *mdtABC* operon was upregulated under stress caused by excess of zinc when conducting the microarray analysis of chemostat-cultured *E. coli* MG1655. Furthermore, *mdt*ABC has been experimentally confirmed to play a role in detoxification of heavy metals, in particular, zinc and copper [47,48] allowing resistance to such metals.

We provide new knowledge on how $bla_{\text{CTX-M-1}}$ expression is influenced by zinc concentrations. The results demonstrate significant changes in mRNA levels depending on zinc concentration, and there was a general tendency for $bla_{\text{CTX-M-1}}$ expression to increase with increasing zinc concentrations. This was seen in both strains tested. The potential reasons might be related to the insertion sequence, IS*Ecp1* (IS1380 family), which is normally located upstream of the $bla_{\text{CTX-M-1}}$ gene [49]. It has been demonstrated that IS*Ecp1* can mobilize the downstream-located $bla_{\text{CTX-M}}$ gene and work as a promoter for its expression [50,51]. Sub-inhibitory zinc and cadmium concentrations can induce the promoter activity of many insertion sequence (IS) families, such as ISRme5 (IS481 family), IS1087B (IS3 family) and IS1088 (IS30 family) [52]. Accordingly, higher zinc concentrations might induce promoter activity of IS*Ecp1* as well, resulting in higher expression of the downstream-located $bla_{\text{CTX-M}}$ gene (Fig. 4). In addition, IS families were associated with gene inactivation resulting in increased zinc resistance. For example, the inactivation of the gene coding for the anti-sigma factor CnrY by IS1087B was shown to cause increase in the transcription of the structural *cnr*CBAT (coding for the resistance-nodulation-division [RND]-driven efflux system CnrCBAT), and this resulted in increased (non-specific) Zn^{2+} efflux in *Cupriavidus metallidurans* AE126 [52]. Accordingly, the IS*Ecp1* might be associated with increase of zinc efflux (e.g. *mdt*ABC) as well, resulting in high tolerance of ESBL-producing *E. coli* to the presence of high zinc concentrations. Besides, it is well known that expression of antimicrobial resistance systems of bacteria can be induced by metals. For example, the multidrug efflux pump genes *mdt*ABC in *Salmonella* can be upregulated by BaeRS (a two-component signal transduction system) in the presence of zinc and copper (Fig. 4) [47], and another efflux pump system AcrAB-TolC, conferring resistance to some antibiotics in *E. coli*, is upregulated by SoxS (a global regulator) in response to copper or chromate (Fig. 4) [53]. Furthermore, some previous studies reported that the expression of antibiotic resistance systems could be induced by specific metals, resulting in increased antibiotic resistance. For example, *E. coli* DH5α and LSJC7 enhanced their resistance to tetracycline when exposed to trace amounts of zinc or copper [41]. In *Pseudomonas aeruginosa*, the resistance to carbapenem antibiotics could be induced by the treatment of zinc and copper [54]. Collectively, the expression of the $bla_{\text{CTX-M-1}}$ gene might be induced as a result of zinc exposure.

There are some limitations in this study. We only conducted experiments with two strains and it would be interesting to investigate the growth of additional ESBL *E. coli* strains, including strains with different combinations of zinc and CTX resistance genes, to have a comprehensive understanding of how zinc affects the growth of ESBL-producing *E. coli*. As CTX like zinc induces $bla_{\text{CTX-M-1}}$ expression [27], it would also be interesting to investigate whether there is an additive effect of zinc and CTX on induction of resistance genes ($bla_{\text{CTX-M-1}}$), and to study the growth of two strains in faecal suspension with traces of antimicrobials together with zinc to find out if induction of CTX-M-1 gives an advantage.

Author contributions

AD conceived the study. SP and AH-F performed the experiments. SP, AH-F, JEO and AD participated in study design and provided critical advice. SP, AH-F, JEO and AD analysed the data,

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and SP wrote the first draft of the manuscript. All authors discussed the results and commented on the manuscript.

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Competing interests

None declared.

Ethical approval

Not required.

Appendix A. Supplementary data

Supplementary material related to this article can be found, in the online version, at doi:https://doi.org/10.1016/j.jgar.2020.06.004.

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